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(54) **Title:** USE OF P–GLYCOPROTEIN (Pgp) INHIBITORS IN THE TREATMENT OF CANCER

(57) **Abstract**

The use of Pgp inhibitors, in particular second and third generation resistance modifying agents in the preparation of medicaments for the treatment of cancer and in a method of treating cancer in human or non–human animal subjects, wherein said cancerous cells are multi–drug resistant (MDR) and the MDR phenomenon is dependent upon over–expression of Pgp.

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Use of P-glycoprotein (Pgp) inhibitors in the treatment of cancer

The present invention relates to the use of one or more P-glycoprotein inhibitors as cytotoxic agents in the treatment of cancer in human and non-human animals. In particular it relates to the use of such agents as cytotoxic agents in the treatment of multi-drug resistant cancer cells.

Failure to achieve complete and durable responses from cancer chemotherapy is a common clinical problem which limits the curative potential of antineoplastic agents. Multidrug resistance (MDR) is believed to be a major cause of such treatment failure and is frequently associated with overexpression of the multidrug transporter P-glycoprotein (Pgp). Pgp is a 170 kDa integral plasma membrane protein capable of drug expulsion and maintenance of tolerable intracellular levels of certain cytotoxic drugs (Endicott and Ling (1989) Ann Rev Biochem 58:137; Juliano and Ling (1976) Biochim Biophys Acta 455:152).

Pgp has a broad specificity for multiple xenobiotics of which many are clinically important anticancer drugs which are structurally and functionally diverse (Mulder *et al.*, (1995) Biochem Pharmacol 50:967). Pgp expression has been shown to correlate negatively with chemosensitivity and survival in many cancer types including leukemias, lymphomas, childhood sarcomas, neuroblastomas and ovarian carcinomas. The MDR phenotype may be present at diagnosis or may be acquired during cancer chemotherapy with drugs that are substrates of Pgp.

Several non-cytotoxic drugs for example, calcium channel blockers, calmodulin antagonists, quinolines and cyclosporins, are also substrates of Pgp and some have been shown to competitively inhibit anti-cancer drug

efflux via Pgp and thereby reverse the phenomenon of MDR experimentally (Ford and Hait (1990) Pharmacol Rev 42:155). These MDR modulators are often classified into a number of categories or generations depending on their chemistry and clinical or pharmacological history of use. First generation resistance modifying agents (FGRMA) are drugs originally developed for therapeutic purposes independent of MDR modulation or Pgp inhibition, for example, calcium channel blockers such as verapamil; immunosuppressive agents such as cyclosporin A (CsA) and FK506; antihypertensive drug analogues such as reserpine and yohimbine; the neuroleptic trifluoperazine and anti-oestrogens such as tamoxifen and toremifene (Dantzig *et al.*, (1996) Cancer Research 56:4171-4179).

Some of the FGRMA's for example, the calcium channel blocker verapamil, are able to modify MDR by interfering with Pgp activity *in vivo* as well as *in vitro* (Tsuruo *et al.*, (1981) Cancer Res 41:1967) but the concentration required is associated with severe cardiac toxicity in patients, obviating its clinical utility in reversing MDR (de Faire and Lundman (1977) Eur J Cardiol 6:195). Likewise, the immunosuppressive agent cyclosporin A (CsA) inhibits Pgp activity in cell lines (Slater *et al.*, (1986) J Clin Invest 77:1405) and in animal models (Slater *et al.*, (1986) Br J Cancer 54:235; Meador *et al.*, (1987) Cancer Res 47:6216) but the immunosuppression combined with nephrotoxic and haemodynamic side effects, prohibits its use in modifying MDR therapeutically.

A new class of chemical agents termed second generation resistance modifying agents (SGRMA) have been developed as successors to the FGRMA's. The SGRMA's lack the original pharmacological properties of the first generation agents, but retain a high affinity for Pgp and the ability to circumvent MDR. Loss of the original pharmacological properties of the FGRMA's,

means that SGRMA's may be used therapeutically to circumvent the MDR phenomenon *in vivo*, without the side effects which result from use of FGRMA's (Dantzig *et al.*, supra).

Whilst the SGRMA class of agents show a high degree of structural variation and need not possess any degree of primary sequence or structural homology, common features between them appear to be a high degree of hydrophobicity, a high affinity for Pgp and the ability to diffuse through the cell membrane. They are thought to function by high affinity binding to the Pgp protein which results in functional interference with the transport pump, thus preventing anti-cancer drug efflux (Dantzig *et al.*, *supra*).

The SGRMA class of agents includes, amongst others, the R isomer of verapamil, non-immunosuppressant cyclosporins such as SDZ PSC 833 (Boesch *et al.*, (1991) Exp Cell Res 196:26-32; Boesch *et al.*, (1991) Cancer Res 51:4226-4233), a yohimbine analogue TMBY (Pearce *et al.*, (1989) PNAS, USA 86:5128-5132); MS-073 (Sato (1991) Cancer Research 51:2420-2424); S-9788 (Pierre *et al.*, (1992) Invest New Drugs 10:137-148); GF120918 (Hyafil *et al.*, (1993) Cancer Res 53:4595-4602) and SDZ 280-446 Loor *et al.*, (1992) Br J Cancer 65:11-18). Derivatives of SGRMA's or further drugs selected for greater affinity for Pgp binding and functional interference are often termed third generation resistance modifying agents (TGRMA) and an example of such an agent is LY335979 (Dantzig *et al.*, *supra*).

These SGRMA's and TGRMA's are highly effective MDR modifiers. They are capable of restoring sensitivity in MDR cell lines to oncolytic drugs without exhibiting any significant cytotoxic effects *per se*, towards sensitive parental cancer cells, resistant daughter cancer cells, or towards normal non-cancerous cells, either *in vivo* or *in vitro*, at clinically useful concentrations.

For example, one of these new agents, the

cyclosporin D analogue SDZ PSC 833, has been reported to have no immunosuppressive or nephrotoxic effects and is 10-fold more potent than CsA with respect to MDR reversal (Boesch *et al.*, (1991) *supra*; Twentyman and Bleehen (1991) Eur J Cancer 27:1639-1642). It has been shown to reverse MDR *in vitro* and increase survival time of mice inoculated with MDR leukaemia cells by increasing the toxicity and activity of adriamycin (Keller *et al.*, (1992) Int J Cancer 50:593-597). SDZ PSC 833 is regarded as having no significant cytotoxic activity *per se* against MDR or non-MDR cancerous cell lines or normal mammalian body cells (Keller *et al.*, (1992) *supra*).

The cyclopropyldibenzosuberane modulator, LY335979 fully restored the sensitivity of a variety of MDR cell lines to vinblastine, doxorubicin (Dox), etoposide and taxol at concentrations ranging from 12 nM to 0.1 μM. It significantly increased the life span of mice bearing multi-drug resistant P388/ADR leukaemia cells when administered in conjunction with Dox or etoposide and enhanced the anti-tumour effect of taxol in an MDR human non-small cell lung carcinoma nude mouse xenograft model. Studies have confirmed that the enhanced cytoxicity observed in dual therapy is due to the MDR modulation effect of LY335979 and not due to any cytotoxic effect of LY335979 *per se* (Dantzig *et al.*, 1990 *supra*).

Similar effects of circumventing the MDR phenotype *in vivo* and *in vitro*, by functionally interfering with Pgp activity, without the manifestation of any cytotoxic effects *per se*, have also been reported for other S/TGRMA's for example, MS073 (Sato *et al.*, 1991 *supra*), S9788 (Pierre *et al.*, 1992 *supra*) GF120918 (Hyafil *et al.*, 1995 *supra*) and SDZ 280-446 (Loor *et al.*, 1992 *supra*).

Surprisingly therefore, the applicants have demonstrated dose dependent, anti-proliferative effects

of second and third generation resistance modifying agents (SGRMA's) against certain MDR neoplastic cell lines when administered in the absence of any conventional anti-cancer drug.

This finding is remarkable because such agents, for example SDZ PSC 833, are widely regarded as having a very low toxic potential. In MDR leukemia cell lines expressing high levels of Pgp, the applicants have demonstrated that SDZ PSC 833 is as potent as the anti-leukemic drug daunorubicin with respect to growth inhibition. However, the antiproliferative effect of these agents which include SDZ PSC 833, LY 335979 and SDX 280-446, unlike daunorubicin, is highly selective, i.e. potent toxic effects were exerted on the MDR-leukemia cells at concentrations which are non-toxic to normal mammalian cells.

By culturing cells in the presence of Pgp inhibitors such as SDZ PSC 833, LY335979 and SDZ 280-446 at different dose levels, the growth of MDR leukaemia cells was inhibited in a dose-dependent manner similar to that of the cytotoxic drug daunorubicin as shown in Fig. 5. For example, for KG 1a/200 MDR leukaemia cells, the GI_{50} values were 260 ng/ml (0.2 μM) and 328 ng/ml (0.3 μM) for SDZ PSC 833 and daunorubicin respectively.

Comparative levels of anti-proliferative activity have been recorded using LY 335979 and SDZ 280-446 on similarly Pgp rich MDR cancer cell lines.

According to one aspect, therefore, the present invention provides the use of a Pgp inhibitor as the sole cytotoxic agent in the manufacture of a medicament for the treatment of cancer in human and non-human animals.

Alternatively viewed, this aspect of the invention provides a method of treatment of cancer in the human or non-human animal body, said method comprising administering a Pgp inhibitor as the sole cytotoxic agent.

Viewed from yet another aspect, the present invention provides the use of a Pgp inhibitor as the sole cytotoxic agent in the treatment of cancer in human or non-human animals.

Generally speaking, in the context of the present invention, a Pgp inhibitor may be defined as an agent capable of specifically modifying Pgp activity without affecting other elements of normal cellular physiology or biochemistry. Although not wishing to be bound by theory, it is believed that the mechanism of action of a Pgp inhibitor includes binding to the 170 kDa membrane bound Pgp protein to cause a functional intervention in the activity of the transporter and preventing the efflux of other Pgp substrates.

As will be discussed in more detail below, in some preferred embodiments the Pgp inhibitors of the present invention are those often referred to in the art as second or third generation resistance modifying agents (S/TGRMA's). Where this is the case, the S/TGRMA's are generally those having Pgp-inhibitory activity and recognised in the art as being capable of circumventing the MDR phenotype when used in conjunction with conventional cytotoxic agents, at concentrations which would not result in non-specific toxicity. When the S/TGRMA is recognised as having the pharmacological ability to circumvent the MDR phenomenon when used in conjunction with conventional cytotoxic agents the invention of the present application is limited to the use of S/TGRMA or a method comprising administering an S/TGRMA as the sole cytotoxic agent. In other words S/TGRMA's are established in the art as a distinct and recognised class of RMA's covering a range of chemically unrelated compounds that may have selective effects on the MDR phenotype of cancer cells. Although preferred, the invention is not limited to these classes of Pgp inhibitors.

Preferably, the Pgp inhibitors useful according to

the invention may be defined as agents, which inhibit the action of Pgp in drug efflux, with high potency, i.e. with potency in the order exhibited by SDZ PSC 833 as described by Boesch *et al.*, *supra*, and other SGRMA's or TGRMA's. However, other Pgp inhibitors of lower potency may also be used, although higher doses may be required.

Despite their activity at the level of Pgp binding, the Pgp inhibitors of the present invention, in particular the high potency Pgp inhibitors including the S/TGRMA's, do not exhibit any significant non-specific toxicity to cells or tissues, e.g. healthy cells or tissues.

Techniques and assays for assessing Pgp-inhibitor potency *in vitro* are known in the art and described in the literature and may readily be used to identify Pgp inhibitors for use according to the present invention. Reference may be made for example to the review article by Ferry *et al.*, in Eur. J. Cancer, 1996, 32A(6): 1070-1081, and the references cited therein. Thus, for example, assays may simply measure the increase in the amount of intracellular cytotoxic drug substrate (ie. Pgp substrate) due to exposure of the cell culture *in vitro* to the Pgp inhibitor (Tsuruo *et al.*, (1981) Cancer Research 41; 1967-1972). Proliferation assays may be performed (Tang *et al.*, (1995) Biochemistry 34, 32-39) and the potency of Pgp inhibitors can be assessed in cell free systems using inside-out vesicles (10VS) from Pgp-expressing cells (Horio *et al.*, (1988) Proc. Natl. Acad. Sci USA 89; 3580-3584). An alternative method of assessing the potency of Pgp inhibition is to measure the potency of association of the inhibitor with Pgp by an equilibrium ligand binding assay in membrane particles, which allows Ki values to be determined (Ferry *et al.*, (1992) Biochem. Biophys. Res. Commun 188; 440-445).

Thus, for example, PSC 833 has been assessed to

have an equilibrium constant Ki value of 35 nM for the inhibition of [^{3}H] vinblastine binding to Pgp (Duran *et al.*, (1990) Proc. Ann. Mtg. Am. Assoc. Cancer Res. 35; A2093). However, the calculated Ki values may depend on the competing substrate used for comparison, and, for example, for PSC 833, competition with the cytotoxic drug daunorubicin yields a higher Ki of 200 nm.

Ki values in the nM range are generally regarded in the art as indicative of a potent Pgp inhibitory effect. Thus, advantageously, Pgp inhibitors for use according to the present, have nM affinity to bind to Pgp e.g. as assessed by competitive binding assay as described by Ferry *et al.*, 1992 (*supra*) using one or more cytotoxic drugs e.g. vinblastine.

In one embodiment of the invention, the Pgp inhibitor is selected to have Ki values for competition with vinblastine and daunorubicin, respectively, in the order observed for PSC 833 ie. a value of Ki $\leq$ 100 nm for vinblastine and/or Ki $\leq$ 300 nm for daunorubicin respectively.

As mentioned above, reference may be made to the literature for sources of appropriate Pgp inhibitors to use according to the invention (see for example the S/TGRMA's and references listed above and also Emmer *et al.*, J. Med. Chem. 1994, 37:1918-1928; EP-A-0363212 (1989); WO92/12132 (1992); Slate *et al.*, (1995) Anticancer Research 15: 1-4 which describes the synthesis and/or chemical structures of, *inter alia*, SDZ PSC 833, SDZ 280-440, TMBY, GB120918, 59788 and M5073).

In preferred embodiments of the invention therefore the Pgp inhibitors used may be selected from the R isomer of verapamil, SDZ PSC 833, TMBY, MS-073, S-9788, GF120918, LY335979, SDZ 280-446, and XR(9051) but all other Pgp inhibitors and resistance modifying agents which fulfill the above criteria are within the scope of the present invention, as are their derivatives e.g. functional derivatives and analogues, and any isomers

(e.g. stereoisomers and/or enantiomers) thereof.

Thus, any Pgp inhibitor may be used for example verapamil and cyclosporin A (CsA).

Also included are the salts of such compounds, including both organic and inorganic salts (e.g. with alkali and alkaline earth metals, ammonium, ethanolamine, diethanolamine and meglumine, chloride, hydrogen carbonate, phosphate, sulphate and acetate counterions). Appropriate pharmaceutically acceptable salts are well described in the pharmaceutical literature. In addition, some of these salts may form solvates with water or organic solvents such as ethanol. Such solvates are also included within the scope of this invention.

Procedures, e.g. assays or tests, for performing binding assays or *in vitro* or *in vivo* studies to determine Pgp inhibitor or RMA activity are known to those skilled in the art and are widely described in the literature, for example in the references describing the T/SGRMA's above. Further reference may also be made to Ferry *et al.*, (1996) (*supra*) and Gaveraux J., Cell. Pharmacol., 2: 225-234 (1991).

The specificity of the dose-dependent anti-proliferative effects of the Pgp inhibitor according to the invention is of importance in that it facilitates the use of Pgp inhibitors at concentrations which are toxic to the cancerous cells at issue and non-toxic to normal cells. As with all chemical agents very high dosages of Pgp inhibitors may produce a degree of cell toxicity. Any such toxicity would however be non-specific and would not be included within the scope of the present invention. Advantageously, therefore, the Pgp inhibitors of the present invention are used at a concentration which exerts a specific cytotoxic effect upon the target cancer cells but essentially no anti-proliferative effects upon normal cells.

Conveniently, therefore, the Pgp inhibitor is

administered to the subject being treated in an amount sufficient to achieve a plasma concentration of not more than 5μM preferably not more than 3μM. With the SGRMA, SDZ PSC 833 for example, the preferred plasma concentration will be less than or equal to 3μM, more preferably, between 1 and 3μM, well beneath the maximum tolerable plasma level.

Preferably, the Pgp inhibitor is used at a level which causes 50% or greater growth inhibition of susceptible cell types, preferably at least 60% growth inhibition, for example as measured by the method of Lehne *et al.*, Ex. Int. J. Oncol. (1994) 4: 1229-1235.

Further studies have revealed that the mechanism of action of the selective cytotoxic effect of the Pgp inhibitors on cancer cells may partly involve the induction of apoptosis (i.e. programmed cell death); when cell preparations treated *in vitro* with a Pgp inhibitor and shown to be susceptible to the anti-proliferative effect are subsequently stained, clear evidence of apoptosis, namely DNA fragmentation, may be seen.

Thus, a preferred Pgp inhibitor for use according to the invention is a Pgp inhibitor capable of inducing apoptosis, e.g. SDZ PSC 833.

As used herein, the term "cancer" includes any neoplastic, malignant or pre-malignant condition, including cancer of any of the tissues or cells of the body. Thus, not only solid tumours are covered, but any cancer of the haemopoeitic system, as well as metastases etc. Preferably however cancers covered by the present invention comprise malignant or anaplastic proliferations of cells.

It should be noted that many of the cancers susceptable of therapy using a Pgp inhibitor may be classified as being MDR on account of Pgp expression by the cells of the cancer. Not all MDR cancers express Pgp however and diverse cellular mechanisms involving,

for example, topoisomerase II, cytochome P450 and glutathione-S-transferase may result in cells exhibiting a multi-drug resistant phenomenon without any involvement from Pgp. Thus, the present invention principally concerns cells expressing Pgp and the MDR phenotype referred to herein is that associated with Pgp expression.

Moreover, some cancer cells may express Pgp and thus be susceptible of therapy with the Pgp inhibitor of the present application but not recognised as being MDR, for example cells expressing Pgp are still susceptible to therapy using cisplatin since the anti-cancer agent cisplatin is not a substrate of Pgp.

Experiments have shown the cytotoxicity observed with the Pgp inhibitors for certain cell lines to be highly potent and selective for the MDR phenotype. For example, in the case of SDZ PSC 833 and the sensitive cell line KG1a/0 and the resistant (MDR) line KG1a/200 (derived from the KG1a/0 cell line), SDZ PSC 833 has a very potent anti-proliferative effect upon the multi drug resistant (MDR) cell line, comparable to the potency of the toxic effect of daunorubicin.

In a preferred aspect, therefore, the target cancer cells to be treated are multidrug resistant (MDR).

Several mechanisms of MDR have been described in murine and human cancer cell lines. As mentioned above, a very common form of MDR is Pgp-mediated increased drug efflux resulting in decreased intracellular drug concentrations.

Experiments have shown that the MDR phenotype of the cancerous cells susceptible to treatment with Pgp inhibitors according to the invention is mediated by over-expression of Pgp. Preferably therefore, the cancerous cells against which the PgP inhibitors are targeted in the present invention exhibit over-expression of P-glycoprotein, (Pgp).

The cancer cells to be treated according to the

invention may be relatively undifferentiated, or poorly differentiated and may exhibit other phenotypic characteistics such as lack of responsiveness to regulatory growth control, decreased adhesiveness and cohesiveness, diminished contact inhibition and density dependent growth inhibition and enhanced invasiveness and capacity for metastasis.

The invention may thus conveniently be applied in the treatment of cancer such as leukaemias, lymphomas, myelomas, sarcomas, pediatric tumors e.g. neuroblastomas, and carcinomas, including cancer of e.g. the breast, ovaries and colorectal cancer. Other cancers include renal cell carcinomas, hepatomas and adreno cortical carcinomas. The cancers may be intrinsically MDR, or the MDR may be induced in initially sensitive cancers.

Preferably, the cancers to be treated are leukaemias, and more preferably MDR leukaemias.

For use according to the present invention, the Pgp inhibitor is conveniently formulated to be in a pharmaceutically acceptable form for administration. By "pharmaceutically acceptable", is meant that the ingredients of the formulation are compatible with each other as well as physiologically tolerable to the patient.

Pharmaceutical compositions according to the present invention may be formulated in any conventional manner as is widely understood in the art. Thus, the Pgp inhibitor used in the invention may be incorporated with one or more conventional carriers, diluents and/or excipients, to produce formulations suitable for patient administration. Preferably, sterile injectable solutions will be used but depending on the nature and site of the cells to be treated, alternative means of drug administration may be used for example, a microemulsion-based drink solution may be formulated to facilitate oral administration. Hence, administration

of the medicament may be by any suitable method known in the art. This will generally be by parenteral means, i.e. intravenous, intramuscular, subcutaneous, intraperitoneal or local injection, although it may also, in an appropriate formulation, be administered orally, rectally, intra-nasally, intra-vaginally, topically or by inhalation, depending on the nature of the condition under therapy.

The precise dosage of the active compound to be administered, the frequency and duration of treatment will, of course, depend on a number of factors, including for example, the size, age and weight of the patient, the precise nature of the cancer under treatment and the route of administration. However, as mentioned above, a preferred dosage is one which is sufficient to achieve a plasma concentration of the Pgp inhibitor of not more than 5μM.

Thus, for example, the SGRMA SDZ PSC 833 may be administered e.g. intravenously, in an effective dose of 2 mg/kg as a loading dose followed by 10 mg/kg/day continuous infusion for 2 days. Clearly however, variations in therapy programmes are possible as determined by the physician and this is standard practice in the medical art.

The cytotoxic effect induced by Pgp inhibitors on cancer cells is a new phenomenon with entirely different features from the previously observed effects of selected Pgp inhibitors for example, the SGRMA's, on cells *in vitro* and *in vivo*. Firstly, there is an ordinary dose-response relationship between Pgp inhibitor concentration and cytotoxicity. Secondly, the cytotoxic effect appears in a low-resistant cell line (3-fold resistance) and thirdly, no non-specific growth inhibitory effects are observed at specific low doses of the drug.

Modern cancer chemotherapy frequently entails serious toxic effects on bone marrow, kidney, heart and

lung. In addition, virtually all cytotoxic drugs cause nausea, vomiting and alopecia. The widespread toxic effects are due to a non-selective cellular influence that affects both neoplastic and normal cells, especially rapidly dividing cells. Pgp inhibitors, in particular S/TGRMA's such as PSC 833, LY335979 and SDZ 280 566, may have very low toxicity potentials and thus have no general effect on normal cell growth and function. The remarkable demonstration of specific toxicity towards cancer cells, especially Pgp dependent MDR cells and in particular leukaemia, means that cancers may be effectively treated with one or more Pgp inhibitors, often as the sole therapeutic agents, that is to say, without the concurrent use of any conventional cytotoxic drugs. Such a treatment regime may thus be aimed at maximal drug effect and not at maximal tolerable dose as required for conventional cytotoxic drugs (i.e. conventional chemotherapy). Side effects are minimized and no drug free periods for normal tissue recovery are required. Cumulative organ specific toxicity would no longer limit the total dose of the anti-cancer agent which may be administered and a superior margin of safety is thus afforded by the present invention.

The invention will now be described in more detail in the following non-limiting Examples; with reference to the drawings in which:

Figure 1

Overlay histograms showing the flow cytometric distribution of Pgp immunofluorescence by MRK16 Pgp-targeting antibody (filled histogram) and IgG2a control antibody (open histogram) in the pair of leukemia cells, KG1a/0 and KG1a/200 the non-MDR parental cell line and the MDR daughter cell line respectively. The percentages of positive cells are given in the upper right corner of each histogram.

Figure 2

Overlay histograms showing the flow cytometric distribution of daunorubicin fluorescence in MDR (KG1a/200) and non-MDR (KG1a/0) leukaemia cells. The open histogram represents the fluorescence of daunorubicin when given alone and the filled histogram represents the fluorescence after co-incubation with SDZ PSC 833.

Figure 3

The dose-response curve for SDZ PSC 833-induced modulation of daunorubicin accumulation in MDR (KG1a/200) and non-MDR (KG1a/0) leukaemia cells. The cells were incubated with 2.5 μg/ml daunorubicin and increasing doses of SDZ PSC 833 for 120 min.

Figure 4

The time course of daunorubicin accumulation in MDR (KG1a/200) leukaemia cells during 180 min incubation. SDZ PSC 833 of various concentrations was added after 30 min, and the accumulation of daunorubicin increased immediately and reached higher steady state levels.

Figure 5

Growth inhibition curves showing that SDZ PSC 833 (circles) and daunorubicin (triangles) exert similar growth inhibitory effects in MDR KG1a/200 cells (A), but only daunorubicin produced significant growth inhibition in non-MDR KG1a/0 cells (B). Each point represent the mean of triplicates ± SD.

Figure 6

Growth inhibition curves for MDR KG1a/200 (triangles) and non-MDR KG1a/0 (circles) cells being exposed to increasing doses of verapamil, each point represents the mean of triplicates ± SD.

Figure 7a and b

Growth inhibition curves showing the effects of three Pgp inhibitors, SDZ 280-446 (open squares); SDZ PSC 833 (closed triangles) and LY 335979 (closed circles) on Pgp expressing MDR leukaemia cell lines K562/138 (a) and KG1a/200(b).

Figure 8

Microphotographs of cytospin preparations of MDR KG1a/200 leukaemia cells treated with SDZ PSC 833 for 96 hours and stained with Papanicolou dyes. The nuclear fragments indicated by the arrows, are typical of apoptosis.

Figure 9

Overlay histograms showing the flow cytometric distribution of fluorescence-labelled 3'-OH ends of DNA (filled histogram) in MDR (KG1a/200) and non-MDR (KG1a/0) leukaemia cells. The percentages of positive cells are given in the upper right corner of each histogram. Note the increased fluorescence intensity and the high positive percentage in the resistant leukaemia cells.

Figure 10

Overlay histograms showing the flow cytometric distribution of FITC-dUTP-labelled DNA fragments is resistant KG1a/200(A) and K562/150(B) cells after treatment with 1 μM of SDZ PSC 833, SDZ 280-446 or LY335979 for 48 hours in comparison with the corresponding wild type non-MDR cells. The column to the right lists the mean channel fluorescence (m.c.f.) values. Kolmogorov-Smirnov statistics revealed that dUTP fluorescence increased significantly compared to non-treated cells (distribution not shown) only for KG1a/200 (D = 0.74, D = 0.58 and D = 0.70 for SDZ PSC 833, SDZ 280-446 and LY335979, respectively) and

K562/150 (D = 0.33, D = 0.33 and D = 0.54) cells.

Figure 11

Overlay histograms showing the flow cytometric distribution of FITC-labelled 3'-OH ends of DNA fragments (FL1) and the PI-labelled DNA (FL2) distribution (inserted) in KG1a/0 cells and the resistant KG1a/200 cells. Treatment with 1 μM SDZ PSC 833 for 48 hours (+) was without effect in KG1a/0 cells (A) but resulting in significantly increased FITC fluorescence due DNA fragmentation (large graph) and DNA tetraploidization (small graph) in KG1a/200 cells (B). Similar results were obtained with SDZ 280-446 and LY335979 and in the resistant K562/150 cells with all three agents (not shown).

Figure 12

Bivariate PI-Annexin V scatterplots demonstrating the differential distributions of apoptotic (lower right quadrant) and nectrotic (upper right quadrant) K562/150 cells. The numerical distributions are given as percentages in each quadrant. Treatment with 1 μM SDZ PSC 833 for 48 hours (B) resulted in a marked reduction of viable cells (lower left quadrant) compared to no treatment (A) and a 4-fold increase apoptotic cells. Similar results were obtained with SDZ 280-446 and LY335979 and in the resistant KG1a/200 cells with all three agents (not shown).

Figure 13

Immunoblots demonstrating the distribution of apoptotic proteins in human leukaemia wild type cells and their resistant sublines (KG1a/200 and K562/150). Lane A represents a positive control for bax and bcl-2. Lane B represents a positive control for bcl-2, bcl-xL and p53. The lanes marked with capital letter T represent cells treated with 1 μm SDZ PSC 833 for 48

hours. The blots were representative for two replicate experiments.

Figure 14

Growth inhibition curves for wild type leukaemia cells (KG1a/0, K562/0) and their resistant sublines (KG1a/200, K562/150) after exposure to increasing doses of SDZ PSC 833, SDZ 280-446 and LY335979. Both MDR variants responded to the three agents with significant growth inhibition. Each point represents the mean of triplicates. The error bars represent 95% confidence intervals.

Example 1

Cell lines

An MDR cell line was developed in the laboratory by subculturing drug sensitive human acute myelogenous leukaemia cells, (KG1a, American Type Culture Collection) consisting of undifferentiated promyeloblasts, in the presence of daunorubicin and vincristine, in stepwise increasing concentrations from 10 to 100 ng/ml each as previously described (Lehne *et al.* (1995) Cytometry 20:228).

The cells were propagated in RPMI 1640 medium (Bio Whittaker, Walkersville, MA, USA), supplemented with 10% foetal calf serum, L-glutamine (0.05 in M/ml), streptomycin (100 pg/ml), penicillin (100 U/ml), and nystatin (40 U/ml).

The cell line acquired increased expression of Pgp compared to the parental cell line. The distribution of Pgp expression was determined by flow cytometric immunofluorescence detection using the anti-Pgp monoclonal antibody MRK16. Specific immunofluorescence was obtained by a three-layer staining technique.

Cell suspensions were washed with PBS/BSA and incubated on ice for 60 minutes with MRK16(25 μg/ml), or MOUSE IgG2a (25 μg/ml) in PBS/BSA. The second and third layer staining protocols were carried out with 100 μl biotinylated horse anti-mouse IgG (1:35 dilution in PBS/BSA) and 100 μl fluorescein isothiocyanate (FITC) conjugated streptavidin (1:35 dilution in PBS/BSA) for 20-30 minutes each respectively, with one PBS/BSA wash between them. Immunofluorescence distributions were generated using a FACScan flow cytometer (Becton Dickinson, San José, CA, USA) with a 15 mW argon ion laser tuned to 488 nm. FITC fluorescence of gated populations was collected through a bandpass filter (FL 1, bandwidth 515 - 545 nm). Calculations of

logarithmically amplified fluorescence values were performed in arithmetic mode using the LYSIS (Becton Dickinson, San José, CA, USA) computer program.

The MDR KG1a/200 cells expressed 7-fold more Pgp than the sensitive parental cells, as shown in Fig. 1.

The primary antibody, MRK16 (Hamada & Tsuruo (1986) Proc Natl Acad Sci USA 83:7785), which was a gift from Professor Takashi Tsuruo (Institute of Molecular and Cellular Biosciences, The University of Tokyo) reacts with a membrane surface domain of Pgp. The corresponding IgG2a isotypic control antibody MOUSE IgG2a, was purchased from Monosan, Uden, The Netherlands.

Chemicals

Verapamil hydrochloride was purchased from Knoll AG (Ludwigshafen, Germany). SDZ PSC 833 was a gift from Sandoz Pharma Ltd. (Basle, Switzerland). Epirubicin was provided by Farmitalia, Carlo Erba (Milan, Italy), daunorubicin by Rhône Poulenc Rorer (Vitry, France), and vincristine was purchased from Eli Lilly & Co. (Indianapolis, IN, USA).

Example 2

Cell growth inhibition and SDZ PSC 833 induced reversal of Pgp dependent drug resistance

Approximately 5 x 10^4 cells were plated in 16 mm-diameter wells (Costar Corporation, Cambridge, MA, USA) and grown in 1 ml of drug-free medium for 24 hours. The wells were then supplemented with daunorubicin or a combination of daunorubicin and SDZ PSC 833 in a certain dose range. Replicate cultures (x3 - x6) were made from each of the dose levels and from untreated controls. The pair of KG1a cells was treated for 96 hours.

Harvested cells were counted in a Coulter Counter ZM (Coulter Electronics Ltd., Luton, England). The dose level required for 50% inhibition of cell growth (GI_{50}) was calculated from linear plots of dose versus cell number. The resistance factor (RF) was defined as the ratio between the GI_{50} values obtained in the resistant and sensitive cells, respectively. By co-incubation with SDZ PSC 833 modulation of growth inhibition was assessed. The modulating factor (MF) was defined as the ratio between the GI_{50} values of multiresistant cells with and without SDZ PSC 833, respectively.

The intracellular drug accumulation is both time- and dose-dependent. Incubation for 120 min with 2.5 μg/ml daunorubicin demonstrated that accumulation in MDR KG1a/200 cells was restricted to approximately half of the drug level in parental KG1a/0 cells as measured by flow cytometry. Co-incubation with 1.0 μg/ml SDZ PSC 833 resulted in 2- and 6-fold increase in daunorubicin fluorescence in KG1a/0 and KG1a/200 cells, respectively as shown in Fig. 2.

The dose-response curve for SDZ PSC 833-induced modulation of intracellular drug accumulation is presented in Fig. 3. The sensitive cells also increased their daunorubicin content upon treatment with PSC 833, but to a much lesser extent (Fig. 3). The resistant cells were highly responsive to dose increments between 100 and 500 ng/ml PSC 833, but further increments produced only an insignificant increase in daunorubicin accumulation.

Intracellular accumulation of anthracycline

The MDR KG1a/200 cells were grown in drug-free medium for 24 hours prior to flow cytometric analysis. A FACScan (Becton Dickinson, San José, CA, USA) flow cytometer, tuned to 488 nm laser excitation wavelength, and running at 15 mW, was used to generate anthracycline

fluorescence. The fluorescence was transmitted through a bandpass filter of 564-606 nm (FL2) and logarithmically amplified. Correlated forward angle (a relative measure of cell size) and right angle (a measure of cell granularity) light scatter measurements were generated to exclude dead cells and debris from analyses. Acquired data from 10,000 events were analysed using the LYSIS (Becton Dickinson, San José, CA, USA) computer program. Changes in the fluorescence intensity of epirubicin and daunorubicin were recorded during incubation with the drugs at 37°C, either alone or together with SDZ PSC 833.

The accumulation of daunorubicin in the resistant KG1a/200 cells was registered repeatedly by flow cytometry for 180 min following the addition of 2.5 μg/ml of the drug. A dramatic increase in drug fluorescence was seen immediately after the addition of SDZ PSC 833 as shown in Fig. 4, and the effect was measurable at such low concentrations as 50 ng/ml (0.04 μM).

Resistance modifying effect of SDZ PSC 833

A measure of daunorubicin cytotoxicity was provided by determination of the drug dose that produced a reduction to 50% of the cell numbers in continuously growing cultures (GI_{50}). There was a 3-fold resistance to daunorubicin in the MDR sublines of KG1a (GI_{50} KG1a/200 : 152 ng/ml, GI_{50} KG1a/0: 52 ng/ml). Comparative experiments were carried out with cytotoxic treatment alone or together with PSC 833 to study the growth inhibitory effect of the combined treatment. In the resistant cell lines SDZ PSC 833 conferred a substantial increase in growth inhibition, and the GI_{50} of KG1a/200 decreased by a factor of 7 (GI_{50} untreated: 238 ng/ml, GI_{50} treated: 35 ng/ml). These effects were accomplished by low-dose treatment with 150 ng/ml SDZ

PSC 833 in KG 1a/200 cells. The resistance to daunorubicin was thus completely eliminated by SDZ PSC 833.

Example 3

Cytotoxic effect of PSC 833

Parental KG1a/0 and MDR KG1a/200 cells were grown in the presence of SDZ PSC 833 at different dose levels for 96 hours. The growth of KG1a/200 cells was inhibited in a dose-dependent manner similar to that of the cytotoxic drug daunorubicin. The GI_{50} values were 260 ng/ml (0.2 μM) and 328 ng/ml (0.3 μM) for SDZ PSC 833 and daunorubicin respectively. In the sensitive KG1a/0 cells the GI_{50} for daunorubicin was 111 ng/ml (0.09 μM). Thus, there was a 3-fold relative resistance to the anthracycline in the MDR phenotype of the leukaemia cells. SDZ PSC 833 did not inhibit the proliferation of the sensitive leukaemia cells, as the cell count remained high throughout the entire dose range of SDZ PSC 833 and never reached GI_{50}. The growth curves for the pair of leukaemia cell lines are shown in Fig. 5.

The effects of the resistance modifying agent verapamil against the MDR subline was also investigated. A growth inhibitory effect in the MDR leukaemia cell line was also noted for verapamil as shown in Fig. 6 with the GI_{50} being 11.6 μM for the resistant cell line.

Example 4

Methods similar to those employed in Example 3 were used to determine the cytotoxic effect of SDZ 280-446; SDZ PSC 833 and LY 336979 on two different Pgp expressing MDR cell lines; K562/138 (Fig. 7(a)) and KG1a/200 (Fig. 7(b)) with the exception that the KG1a/200 cells were cultured to 96 hours prior to

counting and the K562/138 cells were cultured for only 72 hours.

Example 5

Assessments of apoptosis

The assessment of apoptosis was based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of DNA, resulting in the synthesis of a polydeoxynucleotide polymer. Resistant and sensitive leukaemia cells (0.5×10^6 - 1×10^6) were fixed in 1% ice cold paraformaldehyde and subsequently in methanol at -20°C. After washing, the cells were incubated (30 min, 37°C) in a total volume of 50 µl TdT solution (Boehringer Mannheim GmbH, Germany) containing 5 U TdT (100U/ml), 10 µl 5x terminal transferase reaction buffer, 3 µl cobalt chloride (1.5 mM), 0.5 µl biotin-labeled *d*-uridine-5'-triphosphate (dUTP) (10 µM), and 5 µl dithiothreitol (DTT) (0.1 mM) in water. The cells were washed in PBS once and subsequently in PBS containing 0.1% (v/v) Triton X- 100 and incubated (30 min, on ice) with 50 µl of streptavidin-fluorescein isothiocyanate (FITC) 1:50 dilution in PBS with 0.1 % (v/v) Triton X-100 and 3% (w/v) nonfat dry milk. Cells were then washed, incubated (10 min, 20°C) in 500 PI PBS containing 0.1% (v/v) Triton X-100, 5 µg/ml propidium iodide (PI) and 100 µg/ml RNase A, and analysed on a FACScan flow cytometer. The percentage of FITC positive cells represents the percentage of cells in apoptosis and PI staining represents the DNA distribution in the cells.

Cytospin preparations were made by centrifugation of aliquots of approximately 0.1×10^6 cells in 300 µl RPMI and 300 µl fixative (95 % methanol and 5% carbowax 1540) at 1000 rpm for 5 minutes using Shandon Cytospin 2 (Shandon Scientific Ltd., Cheshire, England). Automated

Papanicolaou staining was performed in a Jung Autostainer XL (Leica, Germany). Firstly, carbowax was removed by rinsing successively in 70% and 50% alcohols and distilled water. Secondly, the cells were stained with Harris hematoxylin (nuclear stain) for 3 min, and then rinsed successively with 70% and 96% alcohols. Between the washes, alkalinisation with ammonia in 70% alcohol was performed to develop the blue colour. Thirdly, the cells were stained with Orange G solution (cytoplasmic stain) for 1 min, and then rinsed with 96% alcohol. Last, the cells were stained with EA50 (Light green and Eosin, both cytoplasmic stains) for 2 min followed by rinsing with alcohol, clearing with xylene and mounting.

Numerous fragments of nuclei were clearly visible by microscopy after 96 hours of drug exposure at 37°C as shown in Fig. 8. Fragmentation of DNA is of course a hallmark of the apoptotic process. To verify this finding, the cellular content of DNA fragments was measured by flow cytometry. The results showed that the resistant leukaemia cells were clearly apoptotic as demonstrated by a 3-fold increase in the fluorescence signal from FITC-tagged DNA fragments as shown in Fig. 9. On the other hand, only a very slight increase in DNA fragments was seen in the sensitive parental cells. Additional evidence for SDZ PSC 833-induced apoptosis in the resistant KG1a/200 cells was provided by DNA staining, which revealed the emergence of a subdipoid population not seen in the untreated cells or in treated sensitive cells (not shown). Moreover, the light scattergram of the KG1a/200 cells changed in favour of increased side scatter after SDZ PSC 833 treatment (not shown).

Example 6

Further studies to investigate the relationship

between Pgp inhibitor agents and apoptosis of Pgp expressing MDR cells were conducted using KG1a/200 and parent wild type cells as previously described and a Pgp dependent MDR cell line K562/150 derived from the human chronic cyeloginous K562/0 (ATCC)cell line (Lozzio *et al.*, (1975) Blood 45:321 by multistep selection to be adapted to 138ng/ml vincristin (each K562 cell line was provided by Dr Astrid Gruber of the Karolinska Hospital, Stockholm, Sweden). The cells were maintained as described by Lehne *et al.*, (1995) Cytometry 20:228. The number of doublings (n) were calculated according to the equation:

$$n=3.32.\log(X/X_0)$$

where X_0 is the cell count at seeding time point and X is the cell count on day X of the culture.

TUNEL assay for detection of apoptotic cells

Terminal deoxynucleotidyl transferase (TdT)-mediated d-uridine-5'-triphosphate(dUTP)-biotin nick end-labelling (TUNEL) utilises the ability of the enzyme TdT to catalyse binding of deoxynucleotides to 3'-OH ends of DNA strand breaks. The method used in this study is based on a method of Gorczynca *et al.*, Cytometry 15: 169, (1994) and modified as described by Lehne *et al.* ((1998) Br J Cancer 78(5):593-600). Briefly, cells were cultured for 48 hours in the presence of 1 μM SDZ PSC 833, SDZ 280-446 or LY335979, and then 10^6 cells were fixed in 1% ice cold paraformaldehyde and subsequently in methanol at -20°C before 30 minutes incubation at 37°C in TdT solution (Boehringer Mannheim GmbH, Germany) including biotin-labelled dUTP. Subsequently, the cells were incubated on ice with streptavidin-FITC for 30 minutes, and finally counterstained with propidium iodide (PI) to measure DNA content. Analyses were performed using FACScan and FACSVantage (Becton Dickinson, San José, CA,

USA) flow cytometers. The percentage of FITC positive cells corresponds to the percentage of apoptotic cells and PI red fluorescence corresponds to the DNA content. Doublet discrimination and exclusion of debris and clumps were achieved using red fluorescence pulse-width analyses.

Detection of apoptosis using Annexin V staining

The method is based on the ability of the vascular protein Annexin V to bind phosphatidylserine which is translocated to the external surface of cells undergoing early phases of apoptosis (Vermes *et al.* Annexin V. J Immunol Methods 184-39 (1995). Cell death was assessed using the Annexin V-FITC apoptosis detection kit (Bender MedSystems, Vienna, Austria) according the manufacturers instructions, and counterstaining of the cells with PI was done to identify necrotic cells. Acquisition and analyses were performed using a FACScan flow cytometer and CellQuest computer program.

The results show that the KG1a/200 and K562/150 MDR variants were clearly apoptotic as demonstrated by a 2-3 fold increase in the FITC-fluorescence signal from dUTP-labelled DNA fragments compared to a neglible increase in DNA fragments in the parental cells (Fig. 10). Furthermore, the DNA distributions showed that the population representing the G1 cell cycle phase of treated cells, was reduced six-fold while a huge population (43%) of cycling tetraploid cells, which stained positively for dUTP, emerged (Fig. 11). This tetraploid population was shown to consist of true tetraploid cells and not doublets or clumps, by the use of pulse-processed red fluorescence signals, which allowed for doublet discrimination. Similar results were achieved with SDZ 280-446 and LY 335979 as well (data not shown). To differentiate between necrosis and

apoptosis bivariate plots of annexin V and PI were obtained (Fig. 12), which showed that any of the three modulators reduced the viable population of the MDR variants of KG1a and K562 cells by approximately 30% after 48 hours incubation, and that apoptosis and necrosis both contribute to the increase in cell death.

Immunoblotting

Cell lines which were to be analysed for expression of several proteins involved in the regulation of apoptosis were prepared for immunoblotting as follows: 2-3 x 10^6 cells from each cell line, washed once in PBS, were boiled 5 minutes in sodium dodecyl sulphate (SDS)-sample buffer containing 0.1 mM phenylmethylsulfonyl-fluoride (PMSF). These were then cooled on ice and frozen at -20°C until use. The proteins and Kaleidoscope molecular weight standards (BioRad, Hercules, CA) were first separated by electrophoresis in 8.0% or 12.0% SDS-polyacrylamide gels (Laemmli UK (1970) Nature 227: 680). Immunoblotting was done as described previously (De Angelis *et al.*, (1995) Br J Cancer 72: 370) using the anti-bax (P19), anti-bcl-xL (S18), and anti-bcl-2 (N19) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), the anti-p53 MAb Pab 1801 (Calbiochem, Cambridge, MA), and the anti- p-83 MAb 34C1 (kindly provided by Dr. T. Stokke, The Norwegian Radium Hospital, Oslo, Norway). The amount of p83 was determined on the same blots as a control for gel loading and cell concentration as described (De Angelis *et al.* (1995) Br J Cancer 72: 370. An amplified biotin-streptavidin alkaline phosphatase staining procedure (Biorad, Hercules, CA) was used to detect primary antibodies.

Expression of certain proteins involved in the regulation of apoptosis, p53, bcl-xL, bcl-2 and bax, was assessed using immunoblotting. Neither of the cell

lines expressed the apoptosis transcription factor p53, which promotes apoptosis subsequent to DNA damage (Velculescu *et al*. Clin Chem 42: 858, (1996)). On the other hand, expression of the pro-apoptosis bax protein was moderate in the cell lines. Both the wild type and the MDR variant of KG1a cells expressed high levels of the anti-apoptotic proteins bcl-2 and bcl-xL, whereas both K562 celltypes expressed low levels of bcl-2 (Fig. 13). Interestingly, there was no difference in the pattern of expression within each pair of MDR and wild type cells. Treatment with SDZ PSC 833 did not induce or affect the expression level of these proteins. Thus, selection for drug resistance in the KG1a and K562 cells did not result in altered expression of the anti- or pro-apoptotic proteins tested by immunoblotting.

Further cell growth inhibition assay

Approximately $5X10^4$ cells were plated in 16 mm-diameter wells (Costar Corporation, Cambridge, MA, USA) and grown in 1 ml of drug-free medium for the first 24 hours. The wells were then supplanted with the drug or combinations of drugs in a certain dose range. At least three replicate cultures were made for each of the dose levels and for untreated controls. The KG1a cells were cultured for 96 hours and the K562 cells were cultured for 72 hours ensuring 2-3 doublings. The actual mean number of doublings (standard error of the mean in parenthesis) for KG1a/0 were 3.1 (SE 0.2), that for KG1a/200 were 2.7 (SE 0.2), that for K562/0 were 3.3 (SE 0.2) and K562/150 were 3.1 (SE 0.4). Harvested cells were counted in a Coulter Counter ZM (Coulter Electronics Ltd., Luton, England,). Cell numbers were assessed electronically with a lower threshold set a 7.5 μm particle diameter to discard cell debris and shrunken (apoptotic) cells. The dose level required for 50% inhibition of cell growth (GI_{50}) was calculated from

linear plots of dose versus cell number. The resistance factor (RF) was defined as the ratio between the GI_{50} values obtained in the resistant and sensitive cells, respectively. By co-incubation with either SDZ PSC 833, SDZ 280-446 of LY335979 modulation of growth inhibition was assessed. The modulating factor (MF) was defined as the ratio between the GI_{50} values of multidrug resistant cells with and without the modulating agent, respectively. Each experiment was performed at least three times.

The growth of the KG1a/200 and K562/150 cells was inhibited in a dose-dependent manner by treatment with nanomolar concentrations of either SDZ PSC 833, SDZ 280-446 or LY335979 as shown in Fig. 14, whereas the growth of the corresponding parental lines remained unaffected by equivalent treatment. The half-maximal growth inhibitory doses for the responsive cells are as follows:

	nM GI_{50} for		
	SDZ PSC 833	SDZ 280-446	LY335979
KG1a/200	312 (SE41)	685 (SE51)	66 (SE1)
K562/150	414 (SE50)	578 (SE54)	48 (SE8)

wherein the cells were cultured for 72 (K562) and 96(KG1a) hours with increasing concentrations of each modulator to obtain the concentration required for 50% inhibition of cell growth (GI50). The values are means of four replicate experiments. Standard errors of the mean (SE) are given in parentheses.

Statistics

Comparisons of mean values were performed by unpaired *t* tests using Prism software (GraphPad, San Deigo, CA, USA). *P*-values <0.05 were regarded as

statistically significant. Histogram comparisons were performed by Kolmogorov-Smirnov statistics using CellQuest software. D-values >0.20 were regarded as satistically significant.

Example 7

Two formulations of the Pgp inhibitor, SDZ PSC 833 for intravenous and oral administration are detailed below:

Ingredients of iv and oral formulations of SDZ PSC 833

Concentrate for infusion		Microemulsion-based drink solution	
Supplied as 1 mL, 5 mL & 10 L ampoules		Supplied as 50 mL bottle	
Content	Amount/mL	Content	Amount/mL
SDZ PSC 833	50.0 mg	SDZ PSC 833	100.0 mg
Polyoxyl 35 castor oil (Cremophor EL	600.0 mg	Polyoxyl 40 hydrogenated castor oil (Cremophor RH40)	522.0 mg
Ethanol, absolute	q.s.	Ethanol, absolute	150.0 mg
Nitrogen	q.s.	DL-alpha-tocopherol	1.0 mg
		Propylene glycol	75.0 mg
		Corn oil, interesterified (Labrafil M2125CS)	150.0 mg

Description of Drug Formulations

The active ingredient of SDZ PSC 833 is a cyclic undecapeptide: Cyclo[(N-methyl-3-oxo-5-[1(E)-prpenyl)]-L-leucyl-L-valyl-sarcosyl-(N-methyl)-L-leucyl-L-valyl-(N-methyl)-L-leucyl-L-alanyl-D-alanyl-)N-methyl)-L-leucyl-(N-methyl)-L-leucyl-(N-methyl)-L-valine].

SDZ PSC 833 oral solution is available in 50 mL bottles, and also contains absolute ethanol, Cremophor RH40 (polyoxyl 40 hydrogenated castor oil), and inactive ingredients (interesterified corn oil, propylene glycol, DL-alpha-tocopherol).

The iv formulation contains SDZ PSC 833, absolute ethanol and Polyoxyl 35 castor oil, also known as Cremophor EL, which is a common vehicle for water-insoluble vitamins and drugs including Sandimmun® (cyclosporin A; CsA) and paclitaxel. In the SDZ PSC 833 concentrate for iv infusion, the ratio of Cremophor EL to active drug is 12:1. For comparison, the ratio for paclitaxel is 83:1.

Example 8

The efficacy of SDZ PSC 833 in killing Pgp dependent MDR leukaemia calls was studied *in vivo*. Thirty two NOD-SCID mice (non-obese diabetic/severe combined immune deficient (adenosine deaminase deficient)) received 10^7 MDR KG1a/200 human leukaemia cells as described *supra* intravenously. Half the sample group were treated with PSC 833 and the other half were treated with a placebo. Drug levels in the bloodstream were tested and corresponded with tolerable levels in humans. The PSC 833 treated group develped fewer tumours than the control group and their survival time exceeded that of control animals (P=0.043)(results not shown).

Claims

1. Use of a Pgp inhibitor as the sole cytotoxic agent in the manufacture of a medicament for the treatment of cancer in a human or non-human animal subject.

2. A method of treatment of cancer in a human or non-human animal subject, said method comprising administering a Pgp inhibitor as the sole cytotoxic agent to said subject.

3. Use of a Pgp inhibitor as the sole cytotoxic agent in the treatment of cancer in a human or non-human animal subject.

4. Use or a method as claimed in any one of claims 1 to 3 wherein said Pgp inhibitor is a second or third generation resistance modifying agent.

5. Use or a method as claimed in any one of claims 1 to 4 wherein said Pgp inhibitor has a Ki value of $\leq$ 100 nm for competetion with vinblastine and/or Ki $\leq$ 300 nm for competition with daunorubicin.

6. Use or a method as claimed in any one of claims 1 to 3 wherein said Pgp inhibitor is selected from the R isomer of verapamil, SDZ PSC 833, TMBY, MS-073, S-9788, GF120918, LY335979, SDZ 280-446 and XR(9051) and functional derivatives, analogues and isomers thereof.

7. Use or a method as claimed in any one of claims 1 to 6 wherein said medicament is adapted to be administered to said subject or wherein said Pgp inhibitor is administered to said subject in an amount sufficient to achieve a plasma concentration of not more than 5μM.

8. Use or a method as claimed in claim 7 wherein said plasma concentration achieved is not more than 3 μM.

9. Use or a method as claimed in any one of claims 1 to 8 wherein said Pgp inhibitor is used at a level which causes 50% or greater growth inhibition of susceptible cell types.

10. Use or a method as claimed in claim 9 wherein said % of growth inhibition is at least 60%.

11. Use or a method as claimed in any one of claims 1 to 10 wherein said cancer is a malignant or anaplastic proliferation of cells.

12. Use or a method as claimed in any one of claims 1 to 10 wherein said cancer cells are multi-drug resistant (MDR).

13. Use or a method as claimed in claim 12 wherein said multi-drug resistance is mediated by over-expression of Pgp.

14. Use or a method as claimed in any one of claims 1 to 13 wherein the cells of said cancer are relatively undifferentiated.

15. Use or a method as claimed in any one of claims 1 to 14 wherein said cancer or cancerous cells are leukaemias, lymphomas, myelomas, sarcomas, pediatric tumors or carcinomas.

16. Use or a method as claimed in claim 15 wherein said cancer is a multi-drug resistant leukaemia.

17. Use or a method as claimed in any one of claims 1 to 16 wherein said Pgp inhibitor is capable of inducing

apoptosis.

18. Use or a method as claimed in any one of claims 1 to 17 wherein said medicament is in a form suitable for administration or wherein said Pgp inhibitor is administered parenterally, orally, rectally, intra-nasally, intra-vaginally, topically or by inhalation.

19. Use or a method as claimed in any one of claims 1 to 18 wherein said medicament is adapted to administer SDZ PSC 833 or wherein SDZ PSC 833 is administered intravenously, in an effective dose of 2 mg/kg as a loading dose followed by 10 mg/kg/day continuous infusion for 2 days.

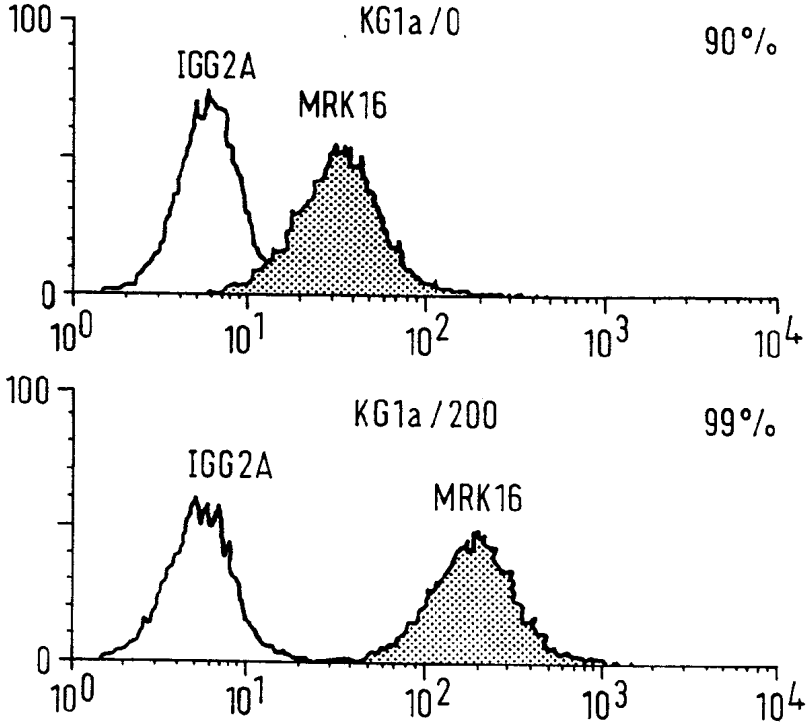

KG1a/0
90%
IGG2A
MRK16
100
0
10^0
10^1
10^2
10^3
10^4
KG1a/200
99%
IGG2A
MRK16
100
0
10^0
10^1
10^2
10^3
10^4

FIG. 1

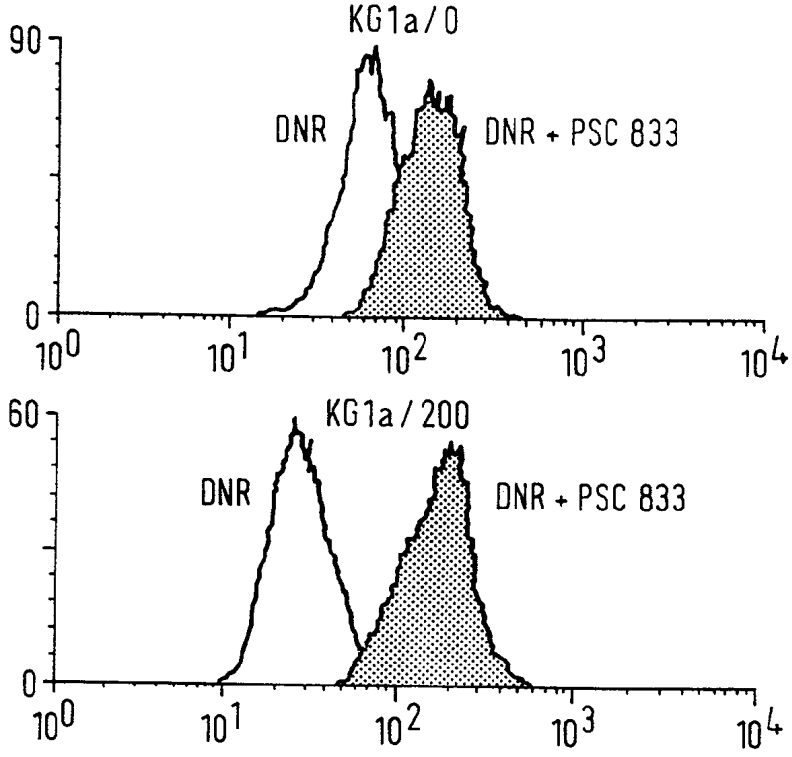

KG1a/0
90
DNR
DNR + PSC 833
0
10^0
10^1
10^2
10^3
10^4
KG1a/200
60
DNR
DNR + PSC 833
0
10^0
10^1
10^2
10^3
10^4

FIG. 2

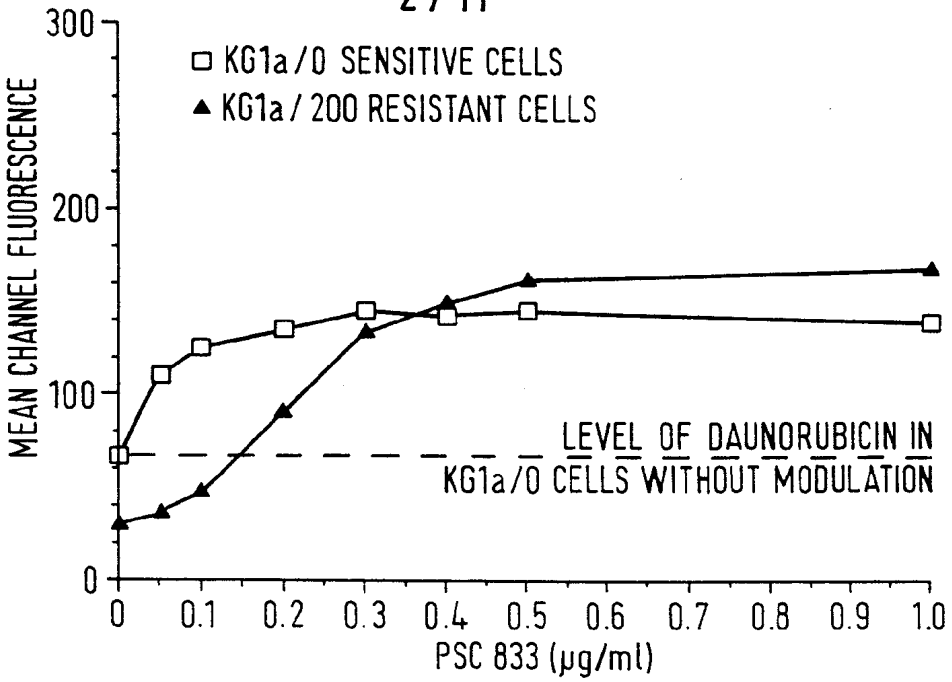

300
200
100
0
MEAN CHANNEL FLUORESCENCE
KG1a/0 SENSITIVE CELLS
KG1a/200 RESISTANT CELLS
LEVEL OF DAUNORUBICIN IN
KG1a/0 CELLS WITHOUT MODULATION
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
PSC 833 (µg/ml)

FIG. 3

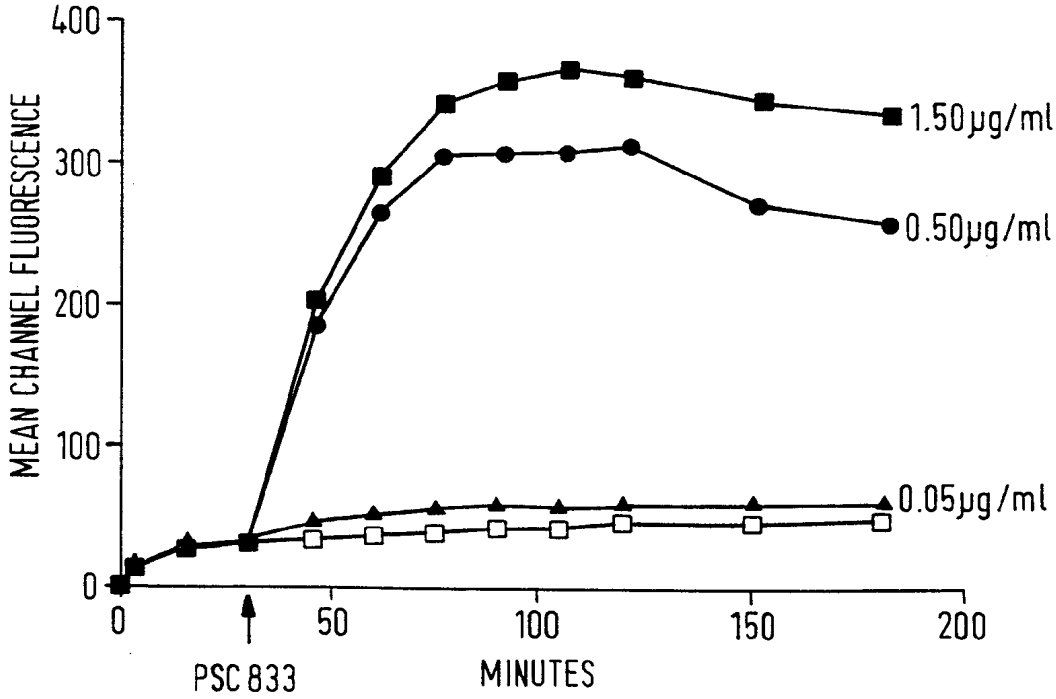

400
300
200
100
0
MEAN CHANNEL FLUORESCENCE
1.50µg/ml
0.50µg/ml
0.05µg/ml
0 50 100 150 200
PSC 833
MINUTES

FIG. 4

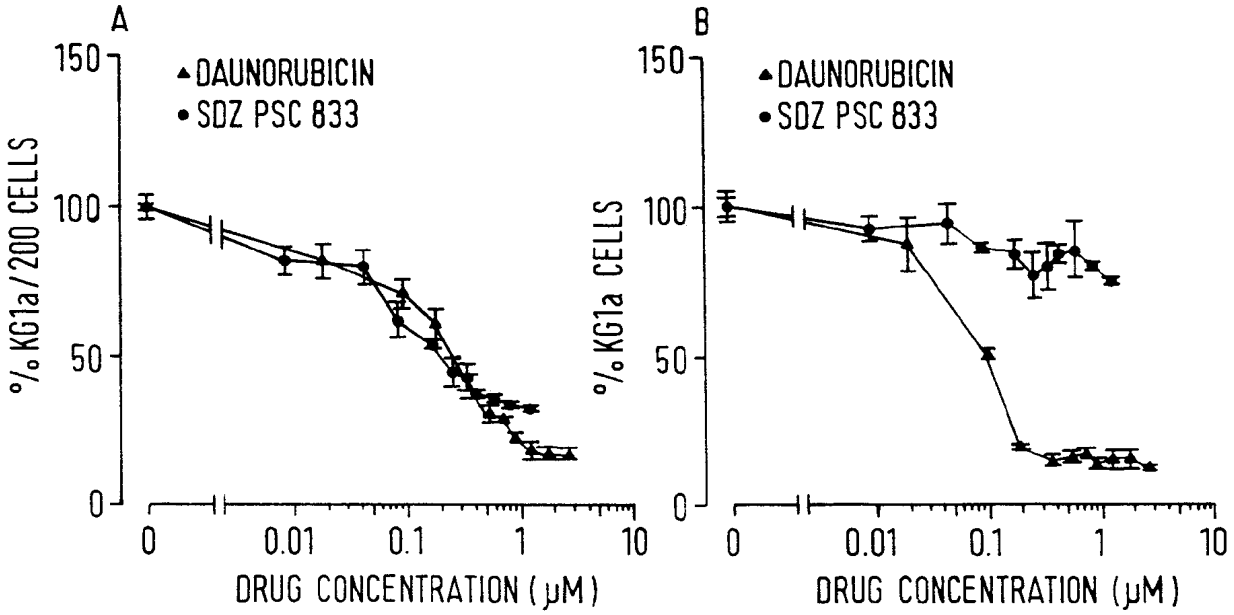

A
B
150
100
50
0
▲DAUNORUBICIN
●SDZ PSC 833
% KG1a / 200 CELLS
% KG1a CELLS
0
0.01
0.1
1
10
DRUG CONCENTRATION (μM)

FIG. 5

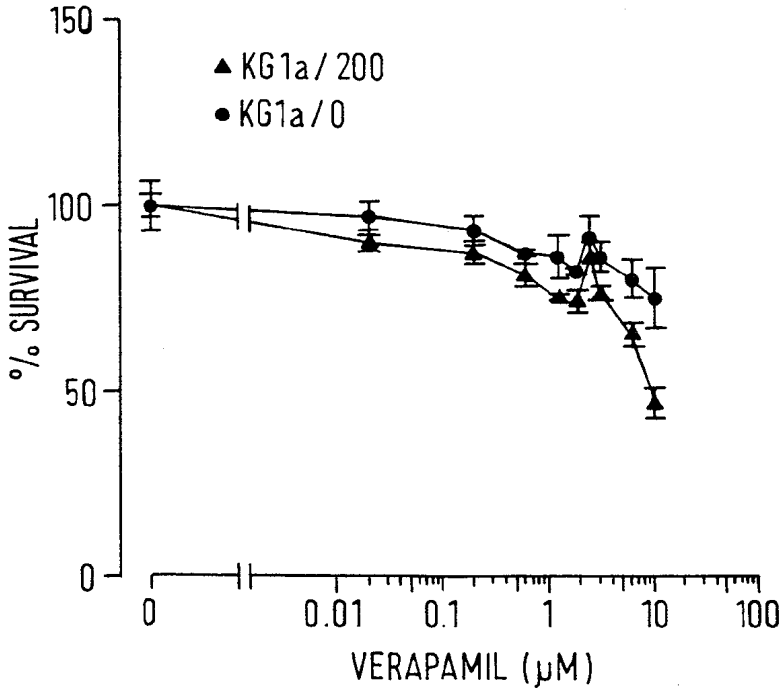

150
100
50
0
▲KG1a / 200
●KG1a / 0
% SURVIVAL
0
0.01
0.1
1
10
100
VERAPAMIL (μM)

FIG. 6

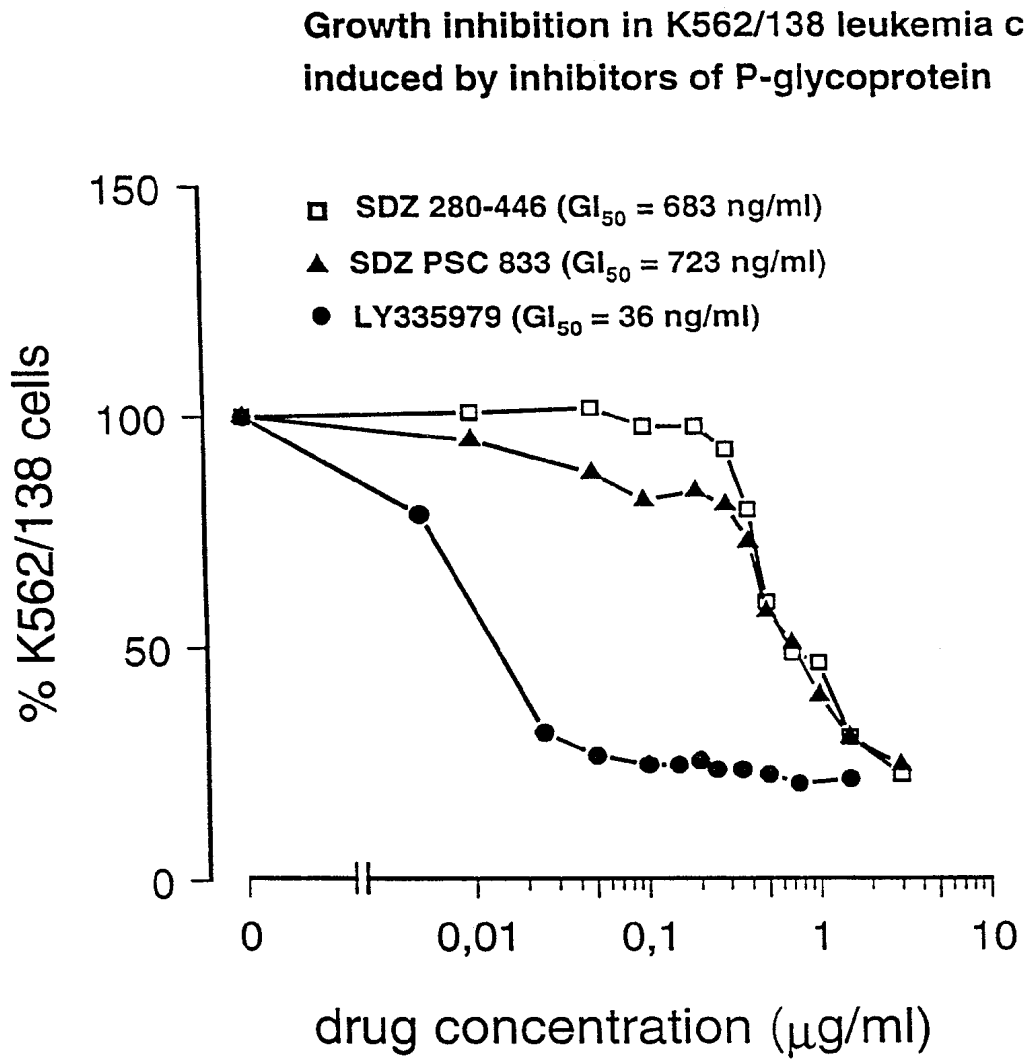

Growth inhibition in K562/138 leukemia cells induced by inhibitors of P-glycoprotein
SDZ 280-446 (GI_{50} = 683 ng/ml)
SDZ PSC 833 (GI_{50} = 723 ng/ml)
LY335979 (GI_{50} = 36 ng/ml)
% K562/138 cells
150
100
50
0
0
0,01
0,1
1
10
drug concentration (µg/ml)

FIG. 7(a)

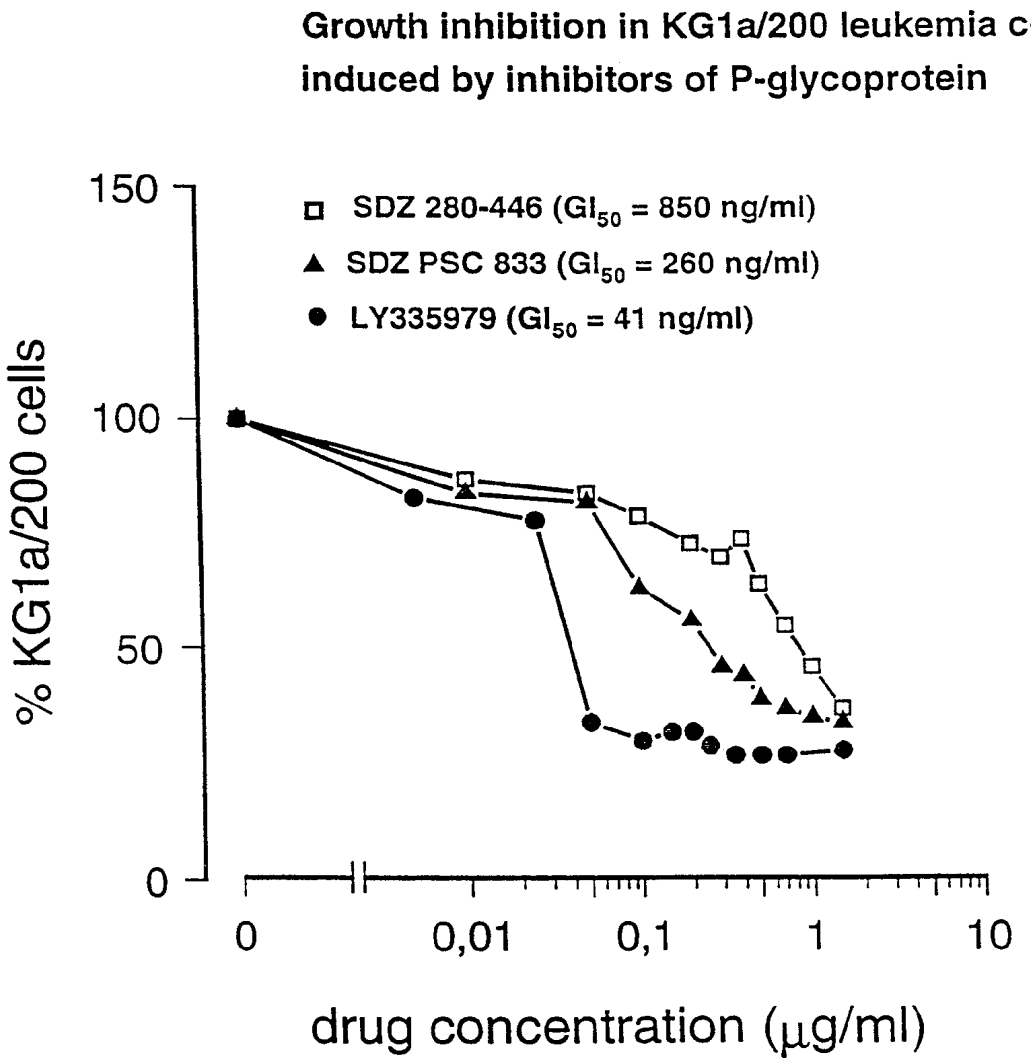

Growth inhibition in KG1a/200 leukemia cells induced by inhibitors of P-glycoprotein
SDZ 280-446 (GI_{50} = 850 ng/ml)
SDZ PSC 833 (GI_{50} = 260 ng/ml)
LY335979 (GI_{50} = 41 ng/ml)
% KG1a/200 cells
150
100
50
0
0
0,01
0,1
1
10
drug concentration (µg/ml)

FIG. 7(b)

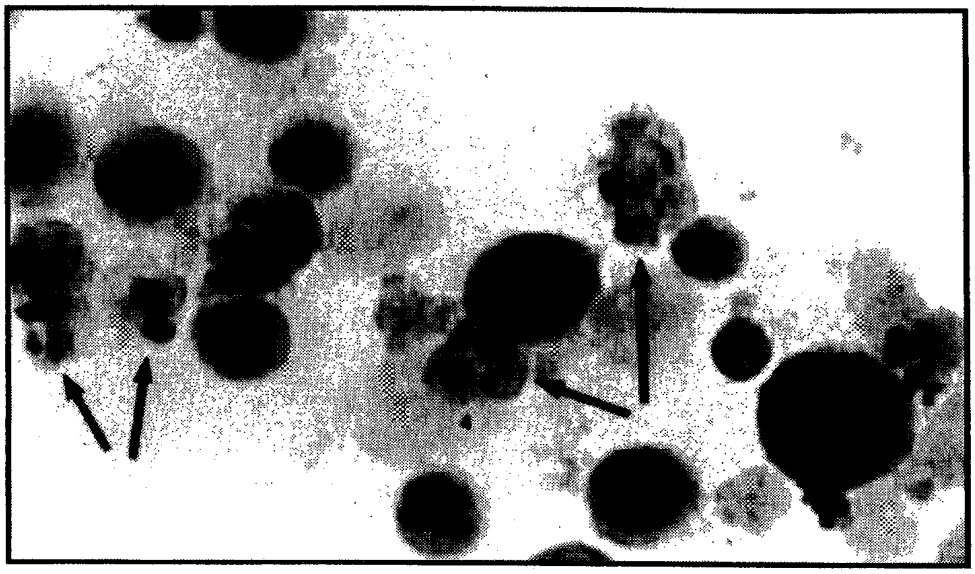


FIG. 8

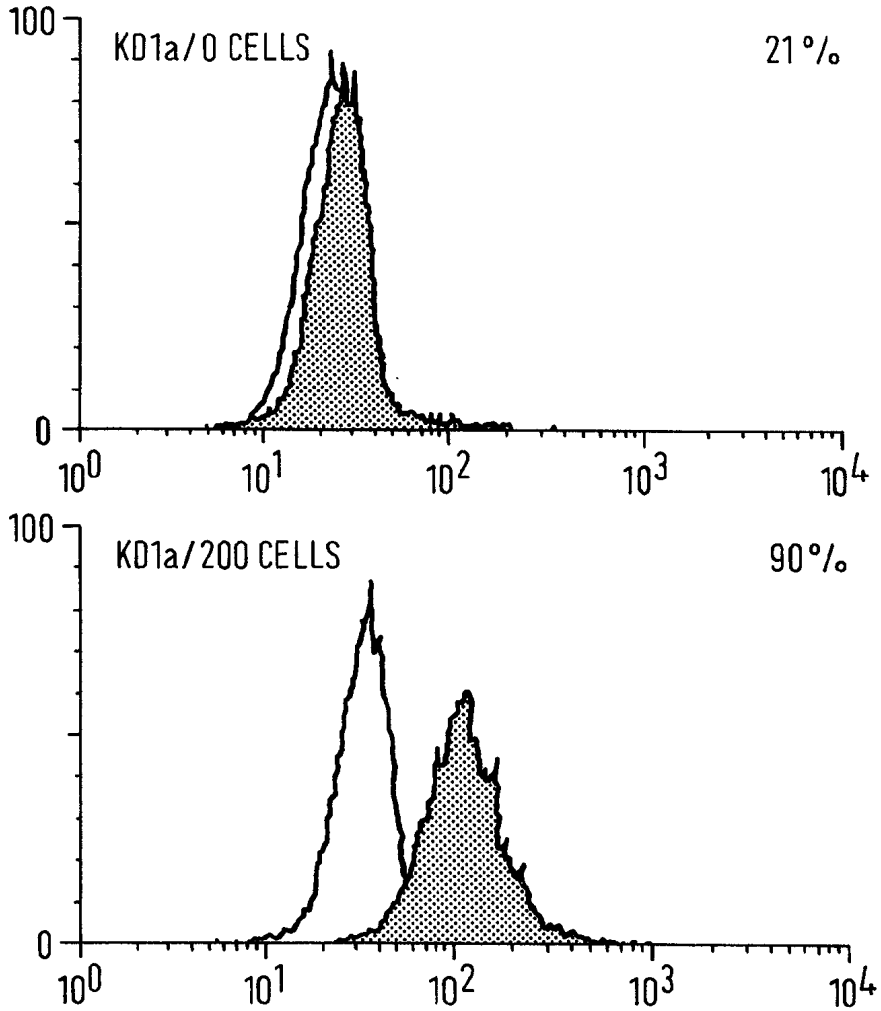

100
KD1a/0 CELLS
21 %
0
10^0
10^1
10^2
10^3
10^4
100
KD1a/200 CELLS
90 %
0
10^0
10^1
10^2
10^3
10^4

FIG. 9

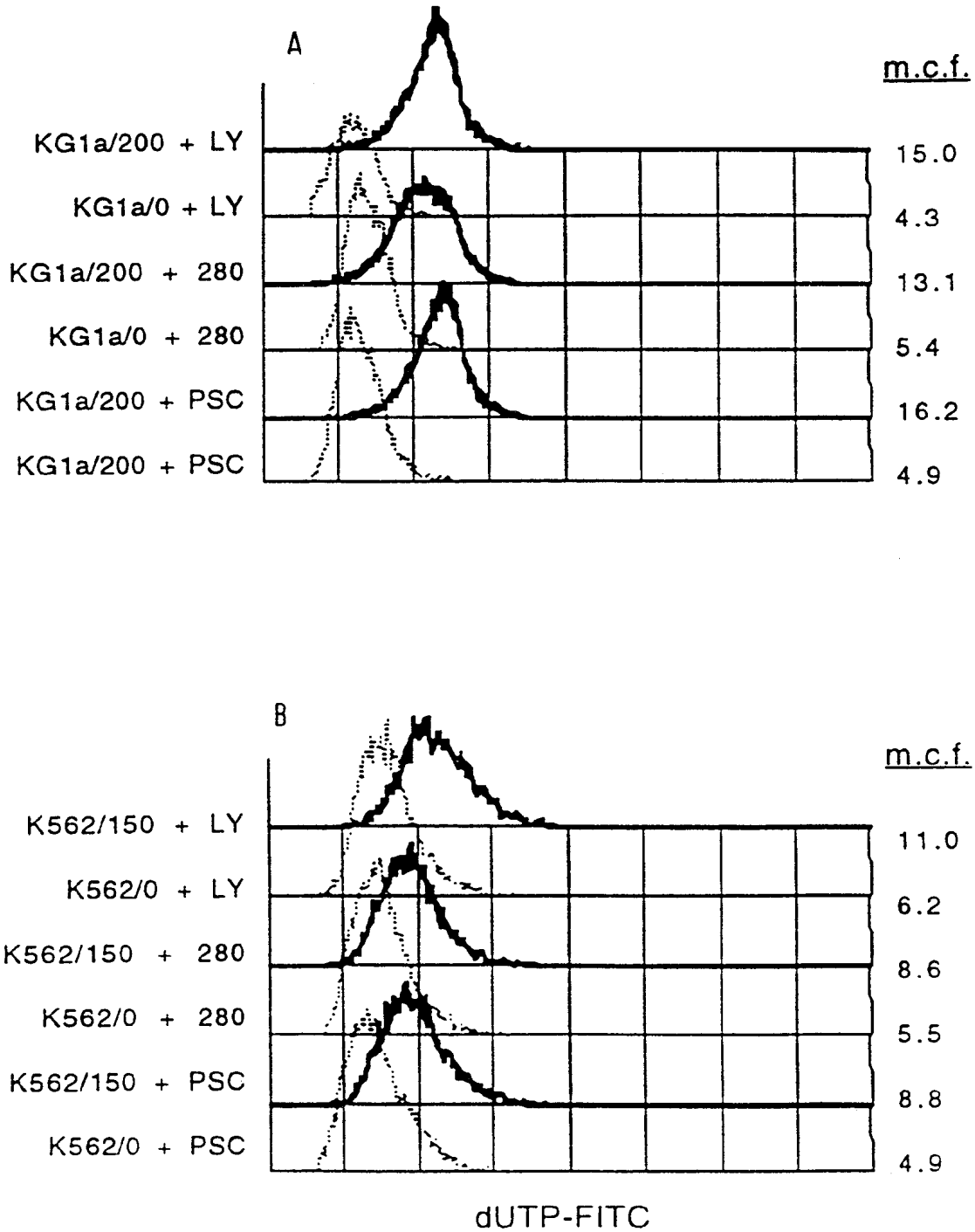

A
m.c.f.
KG1a/200 + LY
15.0
KG1a/0 + LY
4.3
KG1a/200 + 280
13.1
KG1a/0 + 280
5.4
KG1a/200 + PSC
16.2
KG1a/200 + PSC
4.9
B
m.c.f.
K562/150 + LY
11.0
K562/0 + LY
6.2
K562/150 + 280
8.6
K562/0 + 280
5.5
K562/150 + PSC
8.8
K562/0 + PSC
4.9
dUTP-FITC

FIG. 10

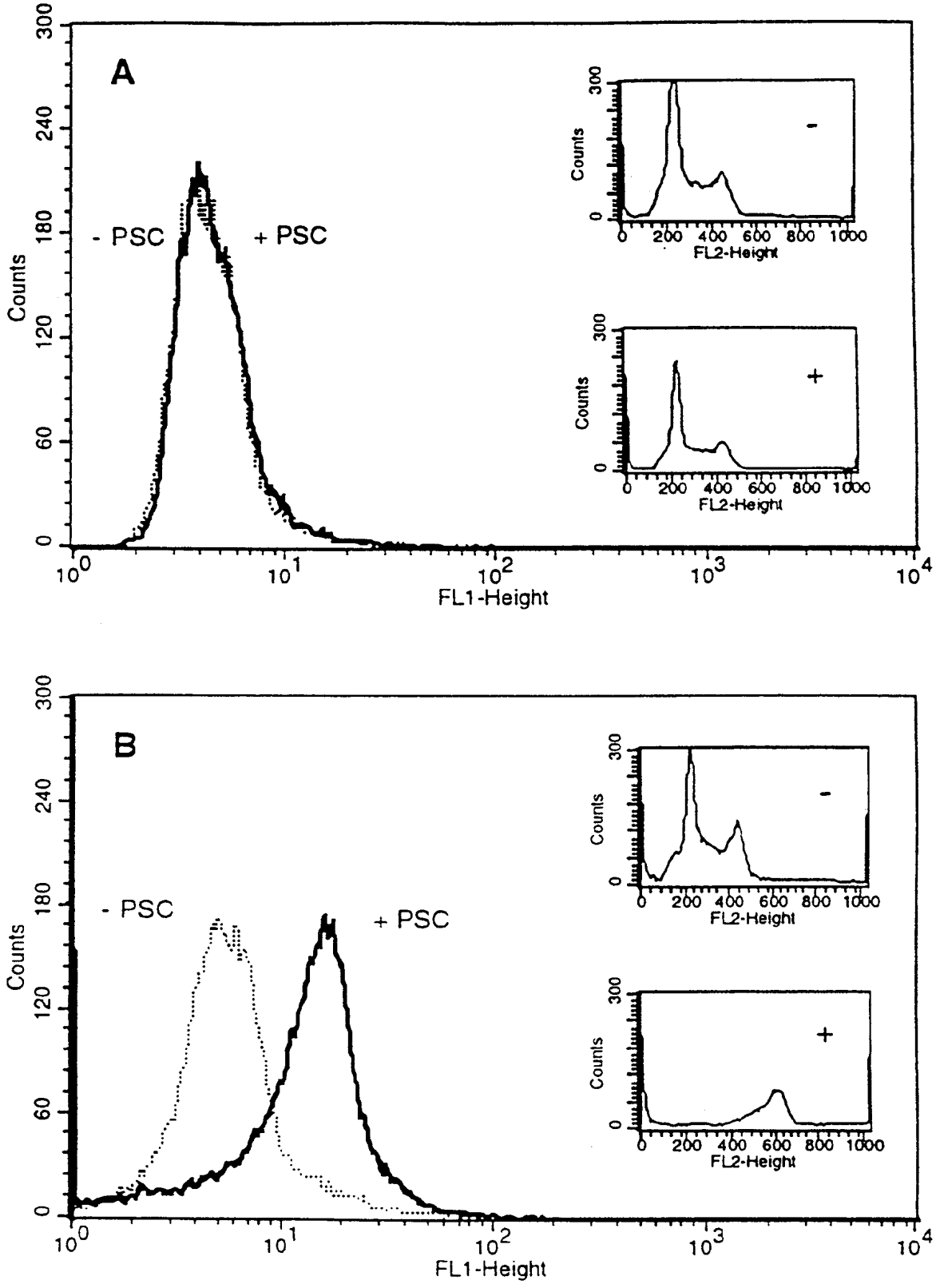
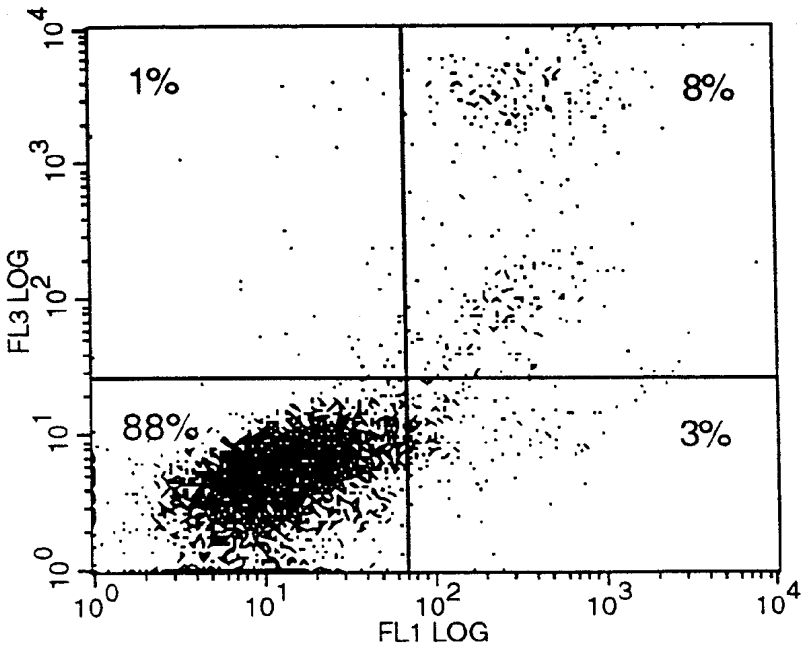

A
- PSC
+ PSC
Counts
FL1-Height
FL2-Height
-
+
B
- PSC
+ PSC
Counts
FL1-Height
FL2-Height
-
+

FIG. 11

A. K562/150 untreated



B. K562/150 treated with SDZ PSC 833

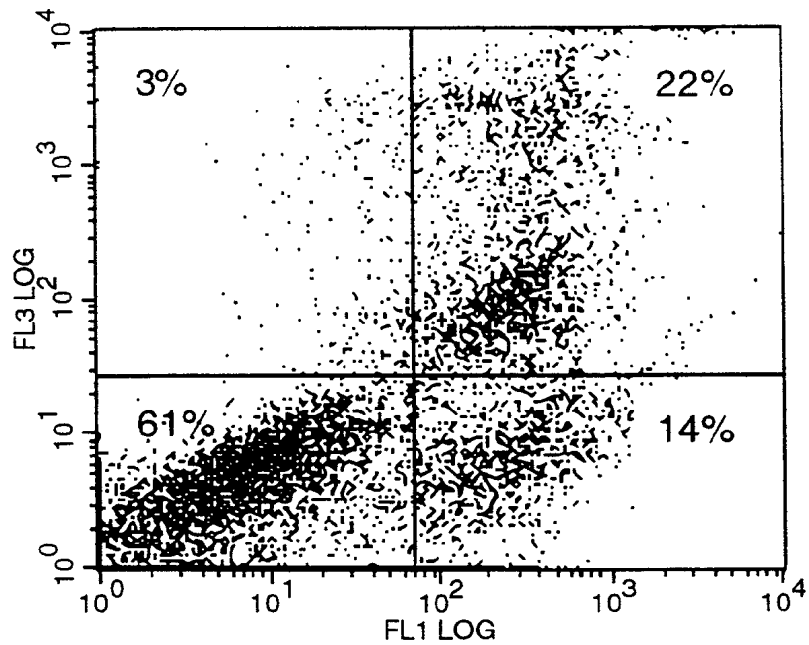


FIG. 12

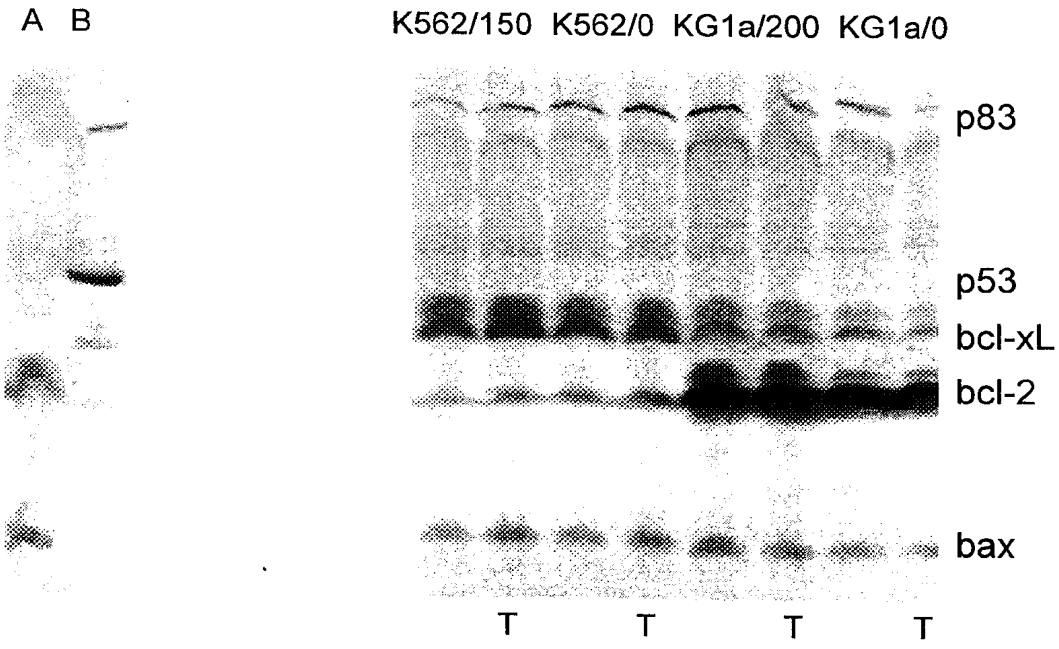


FIG. 13

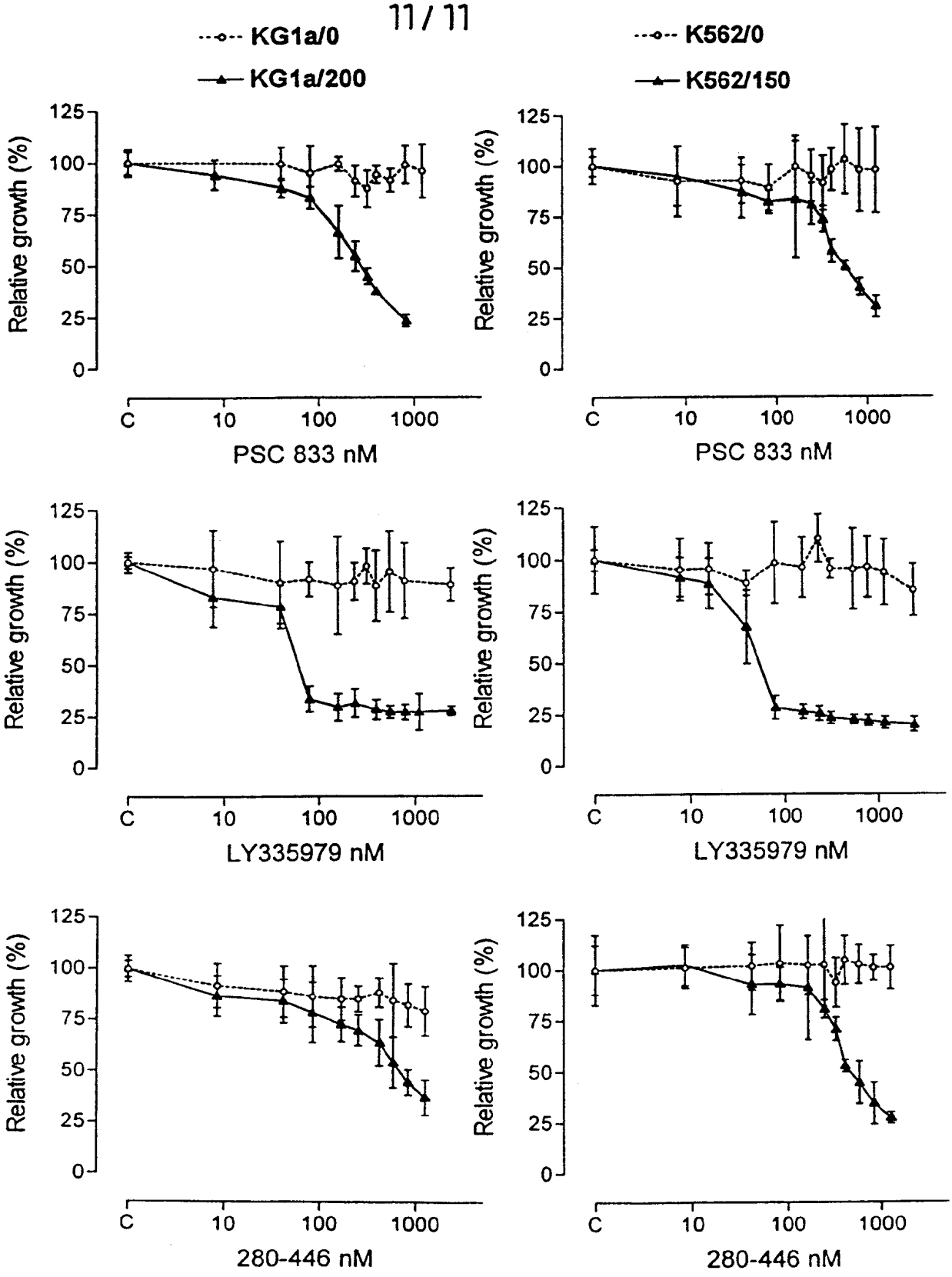

KG1a/0
KG1a/200
K562/0
K562/150
Relative growth (%)
125
100
75
50
25
0
C
10
100
1000
PSC 833 nM
LY335979 nM
280-446 nM

FIG. 14

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 98/03012

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/00 A61K38/13 A61K31/495 A61K31/53 A61K31/47
A61K31/275

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Medline Database Abstract No. 96390602 & Cancer Res., vol. 56, no. 18, 1996 pages 4171-9 XP002092261 cited in the application see abstract ---	1-19
A	Medline Database Abstract no. 92126486 & Br.J.Cancer, vol. 65, no. 1, 1992 pages 11-18 XP002092262 cited in the application see abstract --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 February 1999	18/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Tzschoppe, D

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 98/03012

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication.where appropriate, of the relevant passages	Relevant to claim No.
A	Medline Database Abstract no. 94006239 & Cancer Res., vol. 53, no. 19, 1993 pages 4595-4602 XP002092263 cited in the application see abstract	1-19
A	Medline Database Abstract no. 93053441 & Invest. New Drugs, vol. 10, no. 3, 1992 pages 137-48 XP002092264 cited in the application see abstract	1-19
A	Medline Database Abstract no. 91199092 & Cancer Res., vol. 51, no. 9, 1991 pages 2420-2424 XP002092265 cited in the application see abstract	1-19
A	Medline atabase Abstract no. 91348118 & Exp. Cell Res., vol. 196, no. 1, pages 26-32 XP002092266 cited in the application see abstract	1-19

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